Translation of JP(A) H4-169195

Applied: October 31, 1990
Application No.: H2-295820
Laid-Open: June 17, 1992

TITLE: **ANTI-ED-B MONOCLONAL ANTIBODY**

INVENTOR: Kiyotoshi Sekiguchi, Kooichi Chitani (or Sentani, Senya), Naonobu Hirano, and Tetsuya Tachikawa

APPLICANTS: Fujita Gakuen and Ohtsuka Pharmaceutical Co., Ltd.

1. TITLE OF INVENTION

Anti-ED-B monoclonal antibody

2. PATENT CLAIM

[Claim 1]

Anti-ED-B monoclonal antibody which characteristically recognizes the ED-B amino acid sequences shown by the formula (1) below.

Formula (1):

Glu-Val-Pro-Gln-Leu-Thr-Asp-Leu-Ser-Phe-
Val-Asp-Ile-Thr-Asp-Ser-Ser-Ile-Gly-Leu-
Arg-Trp-Thr-Pro-Leu-Asn-Ser-Ser-Thr-Ile-
Ile-Gly-Tyr-Arg-Ile-Thr-Val-Val-Ala-Ala-
Gly-Glu-Gly-Ile-Pro-Ile-Phe-Glu-Asp-Phe-
Val-Asp-Ser-Ser-Val-Gly-Tyr-Tyr-Thr-Val-
Thr-Gly-Leu-Glu-Pro-Gly-Ile-Asp-Tyr-Asp-
Ile-Ser-Val-Ile-Thr-Leu-Ile-Asn-Gly-Gly-
Glu-Ser-Ala-Pro-Thr-Thr-Leu-Thr-Gln-Gln-
Thr

[Claim 2]

The anti-ED-B monoclonal antibody described in Claim 1, which is obtained by using the fused protein between the ED-B region 91 amino acids and protein A as an immunogen.

3. DETAILED EXPLANATION OF INVENTION

[Industrial Utilization Field]

The present invention relates to the anti-ED-B monoclonal antibody, further in details to the novel monoclonal antibody against the fibronectin (FN), particularly the FN type comprised in cancer organs.

[Conventional Techniques]

The FN was first reported by Morrison, et al., in 1948 [Morrison P. R., et al., J. Am. Chem. Soc., 70, 3103 (1948)] as one of the blood plasma proteins and it is the multi-functional protein which is widely distributed in various organs and body fluid and is known to contribute to the various biological phenomena such as cell transportation, differentiation, multiplication, and canceration as the cell adhesive factor [Kiyotoshi Sekiguchi, Cell Engineering, Vol. 4, No. 6, p485 - 497 (1985)].

Further, the said FN has been known to exist as two molecular types: FN synthesized in liver and existing in blood is called as a plasma FN (pFN) and FN existing on the cultivated cell surface and within the culture solution is called as a cell type FN (cFN). Such the variety in these FN molecules has been clarified to be caused by the alternative splicing of the gene initial transcript product. There are three regions which receive such the alternative splicing: ED-A, ED-B, and IIIcs. By the combination of the expression in these regions, many molecular species are to be generated.

On the other hand, the FN comprised in the cancer organs (below abbreviated as "cancerous FN") exhibits the unordinary high expression of the said ED-B and is known as the FN having ED-B comprising 91 amino acids [Luciano Zardi, et al., The EMBO Journal, Vol. 6, No. 8, p. 2337 - 2342 (1987)].

[Problems to be solved by the Invention]

Under such the condition, this field anticipates the measures to proceed the research of the said cancerous FN at the molecular level and to enable the measurement (detection) and purification specific to this molecular species, and thus to enable the cancer diagnosis.

The purpose of the present invention is to offer the measure to meet the said expectation. In other words, the present invention aims: to offer the monoclonal antibody which specifically recognizes the said ED-B, thus possesses the specific reactivity with the cancerous FN; to offer the peptide related to the said ED-B, particularly the specific peptide which can be the immunogen for the manufacturing of the said monoclonal antibody and which can be a tracer for the measurement of the cancerous FN; and further to offer the technique to measure the desired cancerous FN or ED-B not only in the conventional solid phase system but also in the liquid phase system by utilizing these.

[Methods to Solve the Problems]

According to the present invention, the anti-ED-B monoclonal antibody which characteristically recognizes the ED-B amino acid sequences shown by the formula (1) below, is offered.

Formula (1):

Glu-Val-Pro-Gln-Leu-Thr-Asp-Leu-Ser-Phe-
Val-Asp-Ile-Thr-Asp-Ser-Ser-Ile-Gly-Leu-
Arg-Trp-Thr-Pro-Leu-Asn-Ser-Ser-Thr-Ile-
Ile-Gly-Tyr-Arg-Ile-Thr-Val-Val-Ala-Ala-
Gly-Glu-Gly-Ile-Pro-Ile-Phe-Glu-Asp-Phe-
Val-Asp-Ser-Ser-Val-Gly-Tyr-Tyr-Thr-Val-
Thr-Gly-Leu-Glu-Pro-Gly-Ile-Asp-Tyr-Asp-
Ile-Ser-Val-Ile-Thr-Leu-Ile-Asn-Gly-Gly-
Glu-Ser-Ala-Pro-Thr-Thr-Leu-Thr-Gln-Gln-
Thr

Further the present invention offers the fused protein between the ED-B region 91 amino acids expressed by the said formula (1) and the protein A.

In the present specification above and below, when amino acids, peptides, protective groups, active groups, and others are expressed by the abbreviated codes, the regulation by IUPAC and the commodity codes in this field are used. The expression of the said acids in the base sequence is the same as above.

The said specific anti-ED-B monoclonal antibody offered by the present invention is the antibody which specifically recognizes the ED-B, and is characterized by the possession of the specific reactivity to the ED-B or the FN having the said region, i.e., to the cancerous FN.

Therefore, the antibody of the present invention can be utilized as the specific antibody at the immunity measurement of ED-B or cancerous FN and the easy measurement method with high sensitivity and high precision can be established by this. Further, the establishment of the said measurement method can offer the cancer screening and diagnostic technique and is also extremely useful for the basic research on the carcinogenic mechanism research and analysis.

Further, the said antibody of the present invention is also useful; for the immunological purification of the said ED-B and cancerous FN by, for example, affinity chromatography.

Further, the said specific peptide (ED-B - protein A fused peptide) offered by the present invention is useful as the immunogen for the manufacturing of the anti-ED-B antibody of the present invention and can be also utilized as the tracer (labeled body) at the said measurement method.

Below, the manufacturing method of the antibody of the present invention is interpreted in details.

The antibody of the present invention can be manufactured by the normal method by using the fused protein between the ED-B region 91 amino acids expressed by the said formula (1) and the protein A as an immunogen [Hanfland, P., Chem. Phys. Lipids, 15, 105 (1975); Hanfland, P., Chem. Phys. Lipids, 10, 201 (1976); .Koscielak, J., Eur. J. Biochem., 37, 214 (1978)].

Here, the said ED-B region has been already known and its gene has been determined.

Further in concrete, the fused cell (hybridmer) is formed between the plasma cell (immunocyte) of the mammal immunized by the said immunogen and the plasma cytoma cell of the mammals. Then the clone which produces the desired antibody (monoclonal antibody) which recognizes the ED-B region of FN is selected, and the cultivation of the said clone manufactures the antibody of the present invention.

The antibody of the present invention may be the coarse antibody solution obtained by the said method, i.e., the supernatant of the antibody producing hybridmer culture or mouse's ascites itself. Further it may be the product purified by ammonium sulfate fraction, ion exchange chromatography, and affinity chromatography such as protein A antigen column.

At the manufacturing of the antibody of the present invention, the fused protein between the ED-B region 91 amino acids of the said FN and the protein A used as the antigen is not limited to any specifics as long as it possesses the amino acid sequence expressed by the said formula (1). Examples are the fused proteins of the protein A with any of the followings: cancerous FN prepared from cancer organs; cancerous FN manufactured by the gene recombinant technique; ED-B region of these cancerous FNs or their fragments; and the synthetic peptides having the said specific amino acid sequence. Among them, the particularly desirable one is that obtained by using the ED-B region 91 amino acids of the present invention as the hapten.

The fused protein between the ED-B region 91 amino acids and the protein A may be desirably manufactured through the gene engineering method by utilizing the established cell stain of the cancerous FN having the ED-B region of FN. The details are as following.

In other words, the total RNA is obtained from the cultivated established cell stain which produces the cancerous FN, as the representative example, WI- 38 VA 13 cell which is the strain cell obtained by the transformation (canceration) of the normal diploid fibroblast cell WI- 38 separated form human fetus lung organ with the tumor virus SV 40, by using the guanidine thiocyanate method [Chirgwin, J. M., et al., Biochemistry, 18, 5294 - 5299 (1979)]. Then from this RNA, poly (A^+) RNA is selected by using oligo dT cellulose column, then cDNA which codes the ED-B region is synthesized by using the polymerase chain reaction method [below this is abbreviated as the PCR method, Saiki, R. K., et at., Science, 230, 1350 - 1354 (1985)] according to

the method by Kawasaki and Wang ("PCR Technology", H. A. Erlich, ed., Stockton Press, New York, p 89 - 98 (1980)).

In other words, after synthesizing a single chain cDNA with reverse transcriptase by using a random hexamer as a primer, the Sac I - Pvu II region which codes the ED-B region on FN cDNA may be amplified by the PCR method by using 5'- CAGAGCTCCT-GCACTTTTGA- 3' as the upstream primer and 3'- TGTGACTGTGTTGTTTGCC- 5' as the downstream primer. Two primers used here may not be particularly limited to the said base sequence and any type may be used as long as the targeted Sac I or Pvu II region is comprised. After cleaving the double chain cDNA obtained above at the Sac I and Pvu II, it is inserted to the Sac I - Acc I site of plasmid pGEM 4 (commercially available from Promega) along with the Pve II - Acc I fragment of FN cDNA cut from plasmid pLF 5 comprising FN cDNA [K. Sekiguchi, et al., Biochemistry, 25, 4936 - 4941 (1986)]. As a result, cDNA clone (pGEM B1) which codes the ED-B of FN and its surrounding region can be obtained.

Then from the said pGEM B1, cDNA which codes the region comprising the ED-B is collected as the Eco RI - Pst I fragment. This is inserted to the Eco R - Pst I site of the protein A gene fusion vector, pRIT 2T (manufactured by Pharmacia), and then the desired expression vector pPAB 1 for the fused protein between the protein A and the ED-B, is obtained.

The transformation of the host by the said expression vector may be carried out by the calcium phosphate method by employing, for example, E. coli N 4830 having the λCI_{B57} temperature sensitive repressor (obtained from Pharmacia) as the host [D. Hanahan, D. M. Glover, ed., "DNA Cloning", Vol. 1, p109 - 135, IRC press, Oxford, 1985)]. After cultivating the transformed body obtained above in the LB medium, the cloning is executed by referencing the method by Hanahan and Meselson [Hanahan, D. and Meselson, M., Gene, 10, 63 - 67 (1980)] and the desired protein A - ED-B fused protein positive clone can be obtained.

The production of the aimed fused protein can be carried out by the cultivation of the said positive clone after the isolation and by applying it the heat induction. The obtained protein can be collected as being released from the bacterial bodies by crushing them with ultrasonic crushing. Further, it can be purified with the chromatography utilizing the immunoglobulin insoluble column. The purified desired immunogen is obtained as above.

Here in the said method, the ED-B gene is cloned to pGEM 4 vector after cleaving it into the Sac I - Pvu II fragment which codes the ED-B region and the Pvu II - Acc I fragment downstream therefrom. However, it is not particularly necessary and, for example, the Sac I - Acc I fragment may be employed from the first by being amplified by the PCR method. Further, the said gene can be totally synthesized by the chemical synthesis of the nucleic acid, by following the normal methods such as the phosphite triester method [Nature, 310, 105 (1984)].

At the manufacturing of the monoclonal antibody of the present invention, the mammals which are immunized by the immunogen, i.e., the said fused protein between the protein A and the ED-B region, are not limited to any specific types. However, they should be desirably selected by considering the compatibility with the plasma cytoma cell used for cell fusion, and generally mouse and rat are advantageously employed.

The immunization can be executed by the general method, for example, by administering the said immunogen or the immunogen bound to the carrier (the different type protein having a high antigen property) by using the appropriate binding reagent as described later, to mammals through the intravenous, endermic, hypodermic, or abdominal injection.

In the said manufacturing method of the antigen, natural or synthetic polymer proteins which are commonly used for the preparation of the ordinary antigen are widely utilized as the carrier protein. Examples of the said carrier are: animal serum albumin such as horse serum albumin, bovine serum albumin, rabbit serum albumin, human serum albumin, and sheep serum albumin; animal serum globulin such as horse serum globulin, bovine serum globulin, rabbit serum globulin, human serum globulin, and sheep serum globulin; animal thyroglobulin such as horse thyroglobulin, bovine thyroglobulin, rabbit thyroglobulin, human thyroglobulin, and sheep thyroglobulin; animal hemoglobin such as horse hemoglobin, bovine hemoglobin, rabbit hemoglobin, human hemoglobin, and sheep hemoglobin; animal hemocyanin such as keyhole limpet hemocyanin (KLH); protein extracted from roundworm (ascaris extract, which is described in the followings or which is further purified: JP Kokai 56-16414, J. Immun., 111, 260 - 268 (1973), J. Immun., 122, 302 - 308 (1979), J. Immun., 98, 893- 900 (1967), and Am. J. Physiol., 199, 575 - 578 (1960)); poly- lysine, poly -glutamic acid, lysine- glutamic acid copolymer, and copolymer comprising lysine or ornithine.

As the hapten- carrier bonding reagent, those commonly used for the antigen preparation are widely utilized. The concrete examples are the following dehydration condensation agents: diazonium compounds such as bis- diazodized benzidine (BDB) and bis- diazodized- 3, 3- dianisidine (BDD) which bind tyrosine, histidine, and tryptophan through crosslinking; aliphatic group di- aldehydes such as glyoxal, malone di-aldehyde, glutal aldehyde, succine aldehyde, and adipo- aldehyde which bind amino group and amino group through crosslinking; di-maleimide compounds such as N, N'- o- phenylene dimaleimide and N, N'- m- phenylene dimaleimide which bind thiol group and thiol group through crosslinking; maleimide carboxyl- N- hydroxy succine imide esters such as methamaleimide benzoyl- N- hydroxy succine imide ester, 4- (maleimide methyl)- cyclohexane- 1- carboxyl- N'- hydroxy succine imide ester, N- succinimidyl -3- (2- pyridyl dicyclo) propionate (SPDP) which bind thiol group and amino group through crosslinking; and carbodiimide type such as N, N- dicyclohexyl carbodiimide (DCC), N- ethyl- N'- dimethyl amino carbodiimide, 1- ethyl- 3- di-isopropyl amino carbodiimide, and 1- cyclohexyl- 3- (2- morpholinyl- 4- ethyl) carbodiimide, which are utilized for the ordinary peptide bonding formation reaction binding the amino group and carboxyl group by amide bonding. Further as the said hapten- carrier binding reagent, the combination of diazonium aryl carboxylic acids such as p- diazonium phenyl acetic acid

with the ordinary peptide bonding forming reagent such as the said dehydration condensation agents may be utilized.

The manufacturing method of the antigen utilizing the said hapten, carrier protein, hapten- carrier bonding reagent, and spacer may follow the normal method. The reaction is generally carried out in aqueous solution or in the ordinary buffer solution with pH of 5 to 10, more desirably pH of 6 to 9, at 0 to 40 °C, more desirably at the vicinity of room temperature. The said reaction usually complete in about 2 to 5 hours.

In the said reaction, the usage ratio of the hapten, hapten- carrier bonding reagent, and the carrier may be appropriately determined. Usually, the carried is used at the amount of 0.5 to 5 times in weight, more desirably 1 to 2 times in weight, of the hapten, and the hapten- carrier bonding reagent is used at 1 to 30 times larger molar ratio. By the said procedure, the desired immunogen comprising the hapten- carrier composite in which carrier is bound to hapten directly or through spacer, is obtained.

The antigen obtained by the completion of the reaction is easily isolated and purified by following the normal methods such as the dialysis, gel filtration, and fractional precipitation methods.

In more concrete, the said immunization is executed as following: the immunogen is diluted to the appropriate concentration with physiological salt solution comprising phosphate buffer solution (PBS) or physiological salt solution and combined with the ordinal adjuvant as desired. Then it is administered to the test animal several times every 2 to 14 days. The said administration amount is, for example, about 10 to 100 μg for mouse and 0.2 to 2.0 mg for domestic rabbit. Applicable examples of the said adjuvant are: whooping cough vaccine, complete Freund's adjuvant, and alum.

For the sampling of the antibody, blood sample is collected from the immunized animal after one to two weeks since the said last administration and serum is separated after the centrifugal separation.

Further as the Immunocyte used for the manufacturing of the said monoclonal antibody, the use of the spleen cell collected after about three day since the said last administration is desirable.

The plasma cytoma cell of the mammal used as the other parent cell fused with the said immunocyte may be the various types which are already known such as myeloma cells: p3 / x63 - Ag 8 (x 63) [Nature, 256, 495 - 497 (1975)]; p3/ x63 - Ag8. U1 (p3-U1) [Current Topics in Microbiology and Immunology, 81, 1 - 7 (1978)]; P3/ NS1- 1-Ag4- 1 (NS- 1) [Eur. J. Immunol., 6, 511 - 519 (1976)]; Sp2/0- Ag14 (Sp 2/O) [Nature, 276, 269 - 270 (1978)]; FO [J. Immunol. Meth., 35, 1 - 21 (1980)]; and 210, RCY 3, Ag 1, 2, 3, (Y3) of rat [Nature, 277, 131 (1979)].

The fusion reaction of the said immunocyte and the plasma cytoma cell can be carried out by following the known methods such as the method by Milstein [Method in Enzymology, 73, pp 3 (1981)]. In more concrete, the said fusion reaction can be executed in the ordinary culture under the existence of the ordinary fusion promoters such as polyethylene glycol (PEG) and Sendai virus (HVJ). The culture may be further added with the co-agent such as dimethyl sulfoxide in order to increase the fusion efficiency. Further, the method by electric treatment (electrical fusion) may be appropriately employed. The usage ratio of the immunocyte and the plasma cytoma cell is the same as in the ordinary method. For example, about 1 to 10 times of immunocytes are employed against the plasma cytoma cell. As the medium for the fusion reaction, various types which are ordinarily used for the multiplication of the said plasma cytoma cell, such as the RPMI- 1640 medium, MEM medium, and others generally used as the medium for this type cell, are exemplified. Such the media should be used by eliminating the serum co-solution such as bovine fetus serum (FCS).

The cell fusion is carried out by mixing the certain amounts of the said immunocytes and plasma cytoma cells well within the said medium and by adding the PEG solution preliminarily heated to about 37 °C and having the average molecular weight of, for example, 1000 to 6000, to the medium ordinarily at the concentration of about 30 to 60 w/v%. Then, the desired hybridmer is formed by repeating the following procedures: adding the appropriate media consecutively, centrifuging, and eliminating the supernatant solution.

The separation of the obtained desired hybridmer is carried out by cultivating it in the ordinary culture used for selection, such as the HAT medium (the medium comprising hypoxanthine, aminopterin, and thymidine). The cultivation in the said HAT medium should be carried out for the time period sufficient enough for the cells besides the desired hybridmer (such as non-fused cells) to die out, ordinarily for several days to several weeks. The hybridmer obtained as above is provided for the search of the aimed antibody and for the single cloning by the ordinal limiting dilution method.

The search of the aimed antibody producing strain is carried out by following the various methods generally used for the detection of antibody such as the ELISA method [Engvall. E., Meth. Enzymol., 70, 419 - 439 (1980)], plaque method, spot method, agglutination method, Ouchterlony method, and the radio immunoassay (RIA) method ["Hybridmer Method and Monoclonal Antibody" published by R&D Planning Co., Ltd., pp 30 - 53, March 5, 1982]. For this search, the use of the said ED-B peptide of the present invention is suitable.

The hybridmer which was obtained as above and produces the desired monoclonal antibody of the present invention can be sub-cultivated in the ordinary culture and may be stored for a long time within liquid nitrogen.

The collection of the monoclonal antibody of the present invention from the said hybridmer employs: the method to obtain as the culture supernatant after cultivating the said hybridmer according to the normal method; and the method to obtain as ascites by

administering the hybridmer to the mammal having the compatibility after the multiplication. The former is suitable to obtain the high purity antibody and the latter is suitable for the mass production of the antibody.

Further, the antibody obtained as above can be further purified by the ordinary measures such as salting out, gel filtration, and affinity chromatography.

The anti-ED-B monoclonal antibody of the present invention can be manufactured as above.

Then, the utilization of the antibody of the present invention is described in details. By utilizing the said antibody, the ED-B region of the FN can be easily and specifically purified by the purification measures such as the immune precipitation method and affinity chromatography. Further, the utilization of the present antibody can specifically measure the cancerous FN within the test sample through the immune reaction by using the body fluid as the sample. Examples of the said method are the ordinary immunological measures such as: the ordinal competitive method, radio immunoassay (RIA) by sandwich method, enzyme- linked immunosorbent assay (ELISA), and agglutination method. The operation and procedure of each of these methods may follow the normal method.

In concrete, when the competitive method is executed, the cancerous TN within the test body to be measured and the ED-B from a certain amount of inactivated FN are fused by the reaction with a certain amount of the antibody of the present invention labeled with labeling agent. Then, the bound body of the ED-B of insoluble FN with the labeled antibody is separated from the unbound labeled antibody. Then the measurement of the labeled activity for one of them can quantitatively measure the cancerous FN within the test sample. When the sandwich method is carried out, the measurement substance (sample) is reacted with the antibody of the present invention which is made insoluble, in order to form the ED-B insoluble antibody composite of FN. Then, this composite is reacted with a certain amount of the labeled antibody, and the measurement of the labeled activity for the bound body of this formed composite with the labeled antibody or the measurement of the unbound labeled activity can quantitatively measure the cancerous FN within the test sample as similarly as above.

In the said various test methods, examples for the sample are: blood, urine, and cell sap. Among them, blood, particularly serum or plasma, is desirable.

The antibody of the present invention labeled by the labeling agent and the labeled antibody may be prepared by following the normal method by using appropriate labeling reagent. Examples of the labeling reagents are: radioactive substances such as ^{125}I, ^{131}I, and tritium; and various enzymatic reagents such as gluco-amylase peroxidase (POX), chymotrypsinogen, procarboxy peptidase, glycero- aldehyde- 3-phosphate dehydrogenase, amylase, phosphorylase, alkali phosphatase, D- Nase, P-Nase, β- galactosidase, glucose- 6- phosphate dehydrogenase, and ornithine decarboxylase. The labeling is carried out, in the case of the radioactive iodine, by the

oxidative iodination utilizing chloramine T [see N. H. Hunter and F. C. Greenwood; Nature, 194, 495 (1962), and Biochem. J., 89, 144 (1963)]. And the introduction of enzymatic reagent may be carried out by the ordinarily known coupling methods such as the method by B. F. Erlenger [Acta. Endocrinol. Suppl. 168, 206 (1972)] and the method by M. H. Karol [Proc. Natl. Acad. Sci., USA., 57, 713 (1967)].

Further, the insoluble antibody of the present invention and the insoluble ED-B of FN, such as those physically or chemically solidified on a plate, may be manufactured by physically or chemically binding the antibody of the present invention or the ED-B to the appropriate insoluble carrier. The applicable carriers are: cellulose powder, Sephadex, Sepharose, polystyrene, filter paper, carboxy methyl cellulose, ion exchange resins, dextran, plastic film, plastic tube, nylon, glass bead, net, polyamine- ethyl vinyl ether- maleic acid copolymer, amino acid copolymer, and ethylene- maleic acid copolymer. The said formation of the insoluble body can be carried out by: the covalent bonding method such as the diazo method, peptide method, alkylation method, and the carrier bonding method by cross-linking reagent (glutal aldehyde, hexamethylene isocyanate and so on are used as the cross-linking reagent); various chemical reaction measures such as the carrier binding method by the UgI reaction; the ionic bonding method utilizing the carriers such as ion exchange resins; and the physical adsorbent method utilizing porous glass such as glass beads as a carrier.

The reaction at the said test methods (immune reaction) can be carried out usually at the temperature of at most 45 °C, more desirably from 4 to 40 °C, by taking several to 24 hours.

As described above, the employment of the antibody of the present invention can easily measure the cancerous FN or the FN having ED-B within the sample at a high precision.

The purification system, design of the measurement system, and other various application by utilizing such the antibody of the present invention is apparent for the traders in the field.

[Effects of the invention]

According to the present invention, the anti-ED-B monoclonal antibody of the FN and the fused protein between the ED-B of the FN and the protein A as the immunogen for the manufacturing thereof are offered. The utilization of the antibody of the present invention offers the research on the cancerous FN and the cancer diagnostic method and treatment methods.

[Examples]

Below, in order to interpret the present invention in further details, examples are presented. However, the present invention is not limited to these.

Manufacturing Example 1

Manufacturing of the ED-B/ Protein A fused protein

(1) Preparation of the Sac I - Pvu II fragment comprising the ED-B region of FN

a) Cultivation of cells

In this example, WI-38 VA 13 cells were employed. The said cells are strain cells obtained by transforming the normal diploid fibroblast WI-38 separated from human fetus lung organ by the tumor virus SV 40, and the characteristics were clarified by Girardi, et al. [Ann. Med. Exp. Biol. Fenn., 44, 242 - 254(1966)], and they are deposited to the ATCC as ATCC CCL 75.1.

The said WI 38 VA 13 cells were cultivated by following the description in "Culture of Animal Cells" written by R. I. Freshney (Anan R. Lis, Inc., New York, 1983).

WI 38 VA 13 cells floated by trypsin treatment were sowed to each of 10 sheets of 15 cm cultivating dish (Falcon organ cultivation dish #3025) by 10^6 units, and cultivated at 37 °C for 5 days under the existence of 5% CO_2 by using the DEM medium (Dulbecco's modified Eagle medium, by Gibco) comprising 10% FCS (bovine fetus serum). Then by using rubber policeman, cells were peeled off from the cultivation dish and about 1 g of WI 38 VA 13 cells were collected by centrifuge (500 x g, for 5 minutes).

b) Preparation of cDNA library

About 1 g of the cells obtained in the said section a) was added to Potter type homogenizer having 15 nl **[Note from the Translator- 1]** GTC homogenate buffer solution [5.3 M guanidium thiocyanate, 0.02 M N- lauryl sarcosyl sodium, 0.03 M tri-sodium citrate, 0.8% β- mercapto ethanol, and 0.7% anti-foam 289 (defoaming agent, manufactured by Sigma)].

After 10 back and forth movements, the content was transferred to a beaker, and the shearing was carried out by letting it vigorously pass through the 20 nl syringe with the 22 G injection needle for three times.

By placing about 4 nl of 5.7 M cesium chloride and 0.1 M EDTA into a centrifugal tube, about 8 nl of the said homogenate was layered over and separated by centrifuge at 20 °C and 32000 rpm for 20 hours in order to collect the total RNA.

The total RNA was diluted to at most the concentration of 5 mg/nl, incubated at 65 °C for 7 minutes, then rapidly cooled within ice water for 2 minutes. Then the same amount of the diploid oligo dT bonding buffer solution [1.0 M NaCl and 20 mM tris-HCl, pH 7.5] and $1/100^{th}$ volume of 20% SDS were added and mixed well. Then, this was added to the oligo gT cellulose column (Bio- Rad) equilibrated by the oligo dT bonding buffer solution [0.5 M NaCl and 10 mM tris-HCl, pH 7.5, 0.1% SDS]. The non-adsorbed fraction was further reacted at 65 °C for 7 minutes, rapidly cooled within ice water for 2 minutes, and added to the column again. The column was washed with 10 times larger volume of the oligo dT bonding buffer solution and further washed with 10 times larger volume of the oligo dT washing solution [0.1 M NaCl and 10 mM tris-HCl, pH 7.5, 0.1% SDS]. A^+ RNA bound to the oligo dT cellulose was eluted by the oligo dT elution

solution [10 mM tris-HCl, pH 7.5, 0.05% SDS]. The eluted solution was added with $1/25^{th}$ volume of 5 M NaCl and 2.5 times larger volume of ethanol, mixed well and left at -20 °C for one day and night. Then this was separated by centrifuge at 12000 rpm for 15 minutes to precipitate poly A^+ RNA, which was suspended into 70% ethanol again and similarly separated by centrifuge. After drying the precipitation, the product was dissolved into the appropriate amount of water.

From the poly A^+ RNA obtained by the said method, the region of the FN cDNA which codes the ED-B region was synthesized by using the polymerase chain reaction method according to the method by Kawasaki and Wang ["PCR Technology", H. A. Erlich, ed., Stockton Press, New York, p 89 - 98 (1980)].

c) Synthesis of primer
The following two oligodeoxynucleotide primers were prepared:
the upstream primer (Sac I site): 5'- CAGAGCTCCTGCACTTTTGA- 3'
the downstream primer (Pvu II) site: 3'- TGTGACTGTGTTGTTTGCC- 5'

The said primers were prepared by using the automatic DNA synthesis device (manufactured by Applied Biosystems, Model 380 A) from β- cyano ethyl phosphoamidite derivatives of four types of bases by the solid phase method. The elimination of protection and the freeing from the solid phase carrier of the synthesized oligonucleotide were carried out by heating within concentrated ammonia solution at 55 °C for 10 hours. The synthetic oligonucleotide prepared as above was purified by HPLC and finally about 50 μg of the aimed oligonucleotides were obtained as the upstream primer and downstream primer, respectively.

The obtained purified oligonucleotide was dissolved into the TE buffer solution [10 mM tris- HCl, pH 7.4, 1 mM EDTA] and preserved at -20 °C.

d) Synthesis of a single chain cDNA
Into a 0.5 nl tube (manufactured by Eppendorf), 10 μl of 2 x reaction buffer [40 mM tris- HCl, pH 8.4, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mg/nl nuclease free bovine serum albumin, 2 mM dATP, 2 mM dGTP, 2 mM dCTP, 2 mM TTP, 2 units/nl of RNasin (manufactured by Promega), and 100 pmol random hexamer (manufactured by Pharmacia)] and 9 μl of solution comprising about 1 μg of RNA which was preliminarily processed at 90 °C for 5 minutes were mixed. Then, 1 μg of the reverse transcriptase (about 200 units) originated from mouse Moloney's leukemia virus was added and incubated at room temperature for 10 minutes and further at 42 °C for 30 minutes in order to synthesize a single chain cDNA. The reaction solution was heated at 95 °C for 10 minutes to halt the reaction.

e) Amplification of Sac I- Pvu II fragment
Into 20 μl solution of the single chain cDNA of which reaction was halted by the heat treatment described in the said section d), 80 μl of 1 x PCR reaction buffer [20 mM tris- HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, and 0.1 mg/nl nuclease free bovine serum

albumin] comprising 50 pmol each of the upstream primer and the downstream primer; and 5 units of Taq polymerase (manufactured by Perkin Elmer/Cetus, 1 μl) were added and 100 μl of mineral oil was layered over. Then the heating process of: 1.5 minutes at 95 °C, 3 minutes at 50 °C, and then 3 minutes at 72 °C, was repeated for 35 times, in order to amplify the Sac I- Pvu II cDNA fragment which codes the desired ED-B region. After the completion of the reaction, 10 units of Sac I was added and incubated at 37 °C for 2 hours in order to expose the Sac I site at the 5' side of the amplified SacI- Pvu II cDNA fragment.

For the said reaction solution, the electrophoresis was carried out under the existence of ethidium bromide by using 1.5% agarose gel and by using ϕ x 174 DNA Hae III decomposed DNA fragment as the molecular weight marker. This confirmed the amplification of the Sac I- Pvu II fragment having the desired size of 385 base pairs.

f) Purification of the Sac I- Pvu II fragment

The Sac I- Pvu II fragment separated on the agarose gel according to the said section e) was adsorbed on the DEAE cellulose film (manufactured by S & S, NA 45) by using the method by Dretzen [Dretzen, G. M., et al., Anal. Biochem., 112, 295 - 298 (1981)]. Then, the adsorbed DNA fragment was eluted from the DEAE cellulose film by using the elution buffer [50 mM tris- HCl, pH 8.0, 1 M NaCl, and 10 mM EDTA] and the desired Sac I- Pvu II fragment (about 100 ng) was collected by the cold ethanol precipitation.

(2) Preparation of the FN cDNA Pve II-Acc I fragment

The human fibronectin cDNA clone pLF 5 (20 μg) isolated by Sekiguchi, et al., [Sekiguchi, K., et al., Biochemistry, 25, p 4936 - 4941 (1985)] was dissolved into 50 μl of reaction buffer [10 mM tris- HCl, pH 7.5, 7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2-mercapto ethanol, and 0.01% bovine serum albumin], added with 20 units of Pvu II and Acc I (both manufactured by Takara Shuzo), and reacted at 37 °C for 2 hours. After the completion of the reaction, the electrophoresis using 1% agarose gel was carried out in order to separate the desired Pve II-Acc I fragment (226 base pairs). Then, as described in the said section (1)- f), the desired DNA fragment (about 500 ng) was collected by using the DEAE cellulose film.

(3) Cloning of Sac I- Acc I fragment of FN cDNA to pGEM 4

The 5 μg of pGEM 4 (manufactured by Promega) was dissolved into 20 μl of the reaction buffer [10 mM tris- HCl, pH 7.5, 7 mM $MgCl_2$, 60 mM NaCl, 7 mM 2- mercapto ethanol, and 0.01% bovine serum albumin], added with 10 units of Sac I (manufactured by Takara Shuzo) and 10 units of Acc I (manufactured by Takara Shuzo), and incubated at 37 °C for 2 hours in order to cleave the poly-linker region of pGEM 4 at the Sac I and Acc I sites. After processing the reaction product with phenol, ethanol precipitation caused the cleavage. The plasmid DNA was collected, which was dissolved into 48 μl of the reaction buffer [50 mM tris- HCl, pH 9.0, 0.1 mM $ZnCl_2$, 1 mM $MgCl_2$, and 1 mM spermidine], added with 20 units of bovine small intestinal alkali

phosphatase (manufactured by Takara Shuzo), and heated at 37 °C for 15 minutes then at 56 °C for 15 minutes in order to perform the de-phosphorylation of 5' terminal.

After adding 2.5 µl of 10% SDS, the enzyme was deactivated by heating at 68 °C for 15 minutes and ethanol precipitation was carried out after phenol extraction. As a result, plasmid DNA of which 5' terminal was de-phosphorylated was collected.

Then 20 ng of the said plasmid DNA and 20 ng of each of the cDNA fragments obtained in the said sections (1) and (2) were dissolved into 24 µl of the ligation buffer [66 mM tris- HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM dithio threitol, and I mM ATP], added with 300 units of T4DNA ligase (manufactured by Takara Shuzo), and incubated at 16 °C for 16 hours in order to insert the cDNA fragment from Sac I site to Acc I site which codes the ED-B region of FN into the Sac I- Acc I site of pGEM 4.

Then, 1 µl of this reaction solution was collected, mixed with 100 µl of E. coli HB 101 competent cell (manufactured by Takara Shuzo), and incubated while ice cooling for 30 minutes then at 42 °C for 90 seconds in order to insert the plasmid DNA into E. coli. This was added with 1 nl of the LB medium [1% bacto- tripton, 0.5% yeast extract, and 1% salt] and cultivated at 37 °C for an hour while shaking. Then, 100 µl was collected, sowed on the LB agar plate comprising 50 µg/ml of ampicillin [1.5% bactoagar, 1% bacto- tripton, 0.5% yeast extract, and 1% salt], and incubated at 37 °C for 14 hours to obtain about 200 units of E. coli colonies transformed by plasmid DNA. Among them, 12 units were randomly sampled, cultivated in the LB medium comprising 50 µg/nl of ampicillin, and the plasmid DNA was collected from each colony by the modified method of Birnbim and Doly [Molecular Cloning, A Laboratory Manual, T. Maniatis, et al., ed. p 368 - 369 (1982)]. By the double elimination of Eco RI and Pst I, the plasmid clone (pGEM B1) having the expected insertion sequence of about 600 base pairs was selected.

(4) Collection of Eco RI and Pst I fragments from pGEM B1

a) Isolation of plasmid DNA

E. coli strain comprising the plasmid clone pGEM B1 obtained the said section (3) was cultivated at 37 °C for 12 hours by using 500 nl of the LB medium comprising 50 µg/nl of ampicillin. Then, bacteria were collected by the centrifuge (5000 x g for 10 minutes) and the plasmid DNA was isolated by the alkali bacteriolysis method [Molecular Cloning, A Laboratory Manual, T. Maniatis, et al., ed. 90 - 91 (1982)].

In other words, bacteria were suspended in the buffer solution 1 [50 mM glucose, 25 mM tris- HCl, pH 8.0, 10 mM EDTA] comprising 8 nl of lysozyme (5 mg/nl) and left at room temperature for 5 minutes. Then this was added with 16 nl of 0.2 N NaOH/ 1% SDS solution and quickly mixed, and then bacteriolysis was carried out for 10 minutes under ice cooling. Then 12 nl of ice cooled 5 M potassium acetate solution (pH 4.8) was added and mixed, and further left for 10 minutes under ice cooling.

Then, the centrifuge was carried out at 20000 rpm, for 20 minutes at 4 °C, and 32 nl of the supernatant was transferred into two Corex glass centrifugal tubes by being divided into 16 nl each. Each was added with 10 nl of isopropanol and left at room temperature for 15 minutes, then the plasmid DNA was collected as the precipitate after the centrifuge at 12000 x g, for 30 minutes at 15 °C.

After drying this precipitation by wind, it was dissolved into 8 nl of the TE buffer solution [10 mM tris- HCl, pH 8.0, 1 mM EDTA], further added with 8 g of cesium chloride and 0.4 nl of 1 mg/nl ethidium bromide solution and mixed well. Then the undissolved products were removed by centrifuge at 2000 rpm for 5 minutes at room temperature. The supernatant was transferred to 12 PA seal tube (manufactured by Hitachi Kouki). After filling the top section of the tube with mineral oil, the plasmid DNA band was formed by the centrifuge at 55000 rpm for 16 hours at 19 °C. Then, the plasmid DNA was collected by using an injection needle, and precipitated by ethanol to obtain the desired pGEM B1 plasmid DNA (about 200 μg).

b) Collection of Eco RI- Pst I fragment

The pGEM B1 plasmid DNA (5 μg) obtained in the said section a) was dissolved into the 25 μl Eco RI- Pst I reaction buffer [10 mM tris- HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithio threitol], added with 10 units of Eco RI and 10 units of Pst I, and incubated at 37 °C for 2 hours in order to cleave the plasmid DNA at the Eco RI site and the Pst I site. By the 1.5% agarose gel electrophoresis, the desired Eco RI- Pst I fragment was separated from the obtained reaction solution and the desired DNA fragment (about 300 ng) was collected by the method using the DEAE cellulose film described in the said section (1).

(5) Insertion of the Eco RI- Pst I fragment into pRIT 2T

a) Manufacturing of the plasmid vector

Protein A gene fused vector pRIT 2T (manufactured by Pharmacia, 2 μg) was dissolved into 20 μl of the Eco RI- Pst I reaction buffer, added with 10 units each of Eco RI and Pst I, and incubated at 37 °C for 2 hours in order to cleave the plasmid DNA at the Eco RI - Pst I site. After phenol extraction of the reaction product, the cleaved plasmid DNA was collected by the ethanol precipitation and its 5' terminal was de-phosphorylated by using bovine small intestinal alkali phosphatase by following the method described in the said section (3). After another phenol extraction, the ethanol precipitation resulted in 1 μg of the desired plasmid vector.

b) Insertion of Eco RI - Pst I fragment into the plasmid vector

The pRIT 2T plasmid (20 ng) cleaved by Eco RI and Pst I and having the de-phosphorylated 5' terminal by following the said section a) and 20 ng of the Eco RI - Pst I fragment originated from pGEM B1 prepared in the said section (4) were dissolved into 24 μl of the ligation buffer described in the said section (3), added with 300 units of T4 DNA ligase, and incubated at 16 °C for 16 hours in order to insert the Eco RI - Pst I fragment originated from pGEM B1 into the poly- linker region of pRIT 2T.

c) Preparation of transformer

By using 1 μl of the reaction solution obtained in the said section b) and by following the method described in the said section (3), E. coli HB 101 strain was transformed and about 50 colonies were obtained on 9 cm LB agar plate. Among them, 12 colonies were individually collected at random and cultivated in the LB medium comprising 1.5 nl of ampicillin. Then the plasmid DNA was collected from each colony by the modified method of Birnbim and Doly. The plasmid DNA (about 1 μg) obtained as above was dissolved into the 10 μl of the Eco RI- Pst I reaction buffer, added with 5 units each of Eco RI and Pst I, and incubated at 37 °C for 2 hours. Then, the obtained reaction product was analyzed by the 1% agarose gel electrophoresis and the clone (pPAB1) which produced the 623 base pair Eco RI - Pst I fragment was identified.

d) The E. coli strain having the plasmid pPAB1 identified at the said section c) was cultivated in the LB medium comprising 500 nl of ampicillin, and about 300 μg of the desired plasmid DNA pPAB1 was obtained by following the alkali bacteriolysis method described in the said section (4)- a).

(6) Introduction of the plasmid pPAB1 into E. coli N 4830.

According to the calcium phosphate method by Mandel and Higa [J. Mol. Biol., 53, 154 (1970)], the pPAB1 plasmid DNA obtained in the said section (5) was introduced to E. coli N 4830 (obtained from Pharmacia) by the procedure below.

In other words, E. coli N 4830 was cultivated in 100 nl of the LB medium at 37 °C while shaking and when the bacterial density reached to about 5×10^7/nl, the cultivation was halted and rapidly cooled in the ice bath. After collecting bacteria by the centrifuge at 4000 x g for 5 minutes at 4 °C, the precipitation was suspended into 50 nl of the ice cooled solution of 50 mM calcium chloride and 10 mM tris- HCl (pH 8.0) and left quietly in ice bath for 15 minutes. Then, after the centrifuge at 4000 x g for 5 minutes at 4 °C, the obtained precipitation was suspended again into 7 nl of the ice cooled solution of 50 mM calcium chloride and 10 mM tris- HCl (pH 8.0) and left quietly in ice bath. Into 0.2 nl of the E. coli suspension prepared as above, 10 μl of pPAB1 plasmid solution dissolved in the TE buffer solution (comprising 10 ng of plasmid DNA) was added and left quietly in ice bath for 30 minutes. Then it was heated at 42 °C for 2 minutes within warm water, added with 1 nl of the LB medium, and incubated at 37 °C for an hour. The E. coli suspension obtained as above (100 μl) was sowed to the LB agar medium comprising ampicillin, of which composition was described earlier, and incubated at 37 °C for 14 hours, in order to generate the transformed E. coli colonies on the agar medium.

(7) Isolation of the protein A - ED-B fused protein

After cultivating the transformed body (E. coli N 4830 transformed by plasmid pPAB1) obtained in the said section (6) in 500 nl of the LB medium at 30 °C for 14 hours while shaking, 500 nl of the LB medium preliminarily heated to 54 °C was added and further cultivated at 42 °C for 90 minutes within warm bath while shaking in order to induce the expression of the protein A - ED-B fused protein.

Then, the bacteria were collected by the centrifuge at 5000 x g for 15 minutes at 4 °C, and this was suspended into 100 nl of the ice cooled tris buffer physiological salt water [50 mM tris- HCl, pH 7.6, 150 mM NaCl]. By using ultrasonic crusher (by using Sonifier 250 manufactured by Branson, the three minute treatment at power setting 7 was repeated three times) within ice bath, the protein within the bacteria was released. By centrifuging about 100 nl of this crushed solution (at 16000 x g, for 20 minutes at 4 °C), 95 nl of the supernatant fraction was collected. After diluting this by adding 300 nl of tris buffer physiological salt water, the protein A - ED-B fused protein was adsorbed to the column by using the column packed with about 10 nl of IgG- Sepharose 6 Fast Flow (manufactured by Pharmacia). The said column was washed with each of: 100 nl of tris buffer physiological salt water and 20 nl of 5 mM ammonium acetate solution (pH 5.0) and then the adsorbed protein was eluted by 0.5 M acetic acid solution. The protein A - ED-B fused protein obtained as above was dialyzed by using the tris buffer physiological salt water for two days and nights and about 1 mg of the desired antigen was obtained.

Example 2
Preparation of hybridmer

The purified ED-B - Protein A fused protein (0.05 mg) obtained in Example 1 was diluted by 0.5 nl of PBS and then mixed and emulsified with the same amount of the complete Freund's adjuvant. This (0.2 nl) was endermically administered to male Bal b/c mouse (8 week old). Then, additional administration was carried out for four times with 2 week intervals, and the spleen was collected after three days from the final immunization.

The spleen cells were collected from the said spleen and red blood cells existing in the said cells were liquefied and eliminated by the treatment with 0.83% ammonium chloride solution at 4 °C for 1 to 2 minutes. The cells obtained as above were used as the sensitized lymph cells and washed with the RPMI-1640 medium heated to 37 °C for three times.

Then, mouse myeloma cells [P3U1, see Current Topics in Microbiology and Immunology, 73, p3 (1981)] were sub-cultivated within the RPMI-1640 medium comprising 15% FCS (bovine fetus serum) and further added with 100 μM of 8-azaguanine. This was used as myeloma cell and washed.

The said myeloma cells and the myeloma cells **[Note from the Translator- 2]** were mixed so that their cell number ratio would be 10 : 1 within the 50 nl tube. The obtained cell mixture was centrifuged at 500 x g for 5 minutes, and the supernatant was eliminated completely by using Pasteur's pipette. These operations were carried out within a water bath maintained at 37 °C.

Then 4 nl of 35% polyethylene glycol 1500 (manufactured by Wako Junyaku, below referred to as PEG) was added, stirred slowly for 1 to 2 minutes, and left for 1 minute. Then, 2 nl of the RPMI- 1640 medium not comprising FCS and maintained at

37 °C was added gradually by taking 1 minute and left for another minute. Further 4 nl of the said solution was added and left for 2 minutes and another 4 nl of the said solution was added and left for 4 minutes. Then 8 nl of the RPMI- 1640 medium comprising 15% FCS, 0.05 titer/l of streptomycin sulfate, 60000 U/l of penicillin G potassium, 54 mg/l of gentamicin, and 1 nl of pyruvate (below this is referred to as the complete RPMI- 1640 medium) maintained at 37 °C was added by taking 2 to 3 minutes and the centrifuge was carried out at 500 x g for 5 minutes. After removing the supernatant by aspiration, it was suspended into the complete RPMI- 1640 medium maintained at 37 °C so that the spleen cell concentration would be 1×10^6 units/nl **[Note from the Translator- 3]**. Then this suspension solution was divided into the 96 well plate (manufactured by Coaster) by 0.1 nl and cultivated within the incubator at 37 °C, 5% CO_2, and 100% humidity. Then each well was added with 0.1 nl of the 10% FCS added complete RPMI- 1640 medium, which was added with 1×10^{-4} M of hypothanthine, 4×10^{-7} M of aminopterin, and 1.6×10^{-5} M of thymidine (below, this is referred as the HAT medium). Then, 0.1 nl of the supernatant was aspirated at the second day and the third day and the new HAT medium (0.1 nl) was added for the solution exchange. Then, the said solution exchange was carried out every two to three days. At the 6^{th} day, the supernatant was similarly aspirated and replaced with the complete RPMI- 1640 medium added with 1×10^{-4} M of hypothanthine and 1.6×10^{-5} M of thymidine (below, this is referred to as the HT medium). Then the multiplication was maintained in the complete RPMI- 1640 medium.

After the cell fusion by the said operation, the colony was observed by the naked eye after 10 to 14 days. When the cells occupied $1/4^{th}$ of the 96 well plate bottom surface, the culture supernatant was tested by the enzyme- linked immunosorbent assay (ELISA) method by using the human placenta originated FN maintaining the ED-B, as the antigen. From the well which became positive, the cloning of the hybridmer was immediately carried out by the limiting dilution method [Method in Enzymology, 73, 3 (1981)].

In other words, by using 20 nl of the 10% FCS added RPMI- 1640 medium which was prepared to comprise 1×10^8 units **[Note from the Translator- 3]** of the Bal b/c type mouse thymocyte, the hybridmer was sowed to 6 well plate by 0.2 nl so that its concentration would be 3 units/ well, 1 unit/ well, and 0.3 unit/ well, and the cloning was carried out to establish the desired hybridmer.

The said cloning utilized the reactivity with the cancerous FN purified from the culture supernatant of the cell WI- 38 VA 13 which is the human normal fibroblast WI-38 deteriorated by tumor virus SV 40 and the placenta originated FN, as the index. The said cloning was carried out four times while confirming no reactivity with the plasma type FN and four hybridmer strains which produce the monoclonal antibody of the present invention having the desired reaction specificity were obtained.

These were called as "OAL- TFN- 01" to "OAL- TFN- 04", respectively.

The clones "OAL- TFN- 01" to "OAL- TFN- 04" obtained above were cultivated in the complete RPMI- 1640 medium under the condition of 5 % CO_2, at 37 °C for 96 hours. The culture solution was centrifuged at 3000 rpm for 10 minutes and the culture supernatant comprising the desired monoclonal antibody was obtained.

Among the obtained clones, one strain (hybridmer producing the antibody of the present invention, OAL-TFN-01) was selected.

The said monoclonal antibody producing cell was deposited to the Micro-organism Industrial Research Center, Industrial Technology Agency, Ministry of International Trade and Industry, with the code of "OAL- TFN - 01" and its deposit number is "Micro-organism Industrial Research Center Bacterial Deposit No. 11540 (FERM P- 11540)".

The said clone OAL- TFN - 01 (1×10^6 units **[Note from the Translator- 3]**) was abdominally administered to the Bal b/c mouse which was preliminarily inoculated with pristane (manufactured by Aldrich). After 10 to 14 days, the accumulated ascites was collected and the ascites containing the antibody of the present invention was obtained.

The antibody within the said ascites was purified by using the gel chromatography (with Sephacryl S- 300) and anion exchange chromatography (with Q-Sepharose) and the purified antibody OAL- TFN - 01 was obtained.

Below, the characteristics of the monoclonal antibody of the present invention obtained in the said example are shown as Example 3.

Example 3
Characteristics of the antibody of the present invention

(1) Subclass of the antibody

Mouse monoclonal antibody subclass identifying kit (manufactured by Bio- Rad) was used to determine the subclass of the antibody of the present invention.

As a result, the subclass of the said antibody was IgM.

(2) Antibody producing level

By centrifuging the culture supernatant obtained Example 2, its supernatant was cultivated in vitro by using the 10% FCS added RPMI- 1640 medium at 37 °C, with 5% CO_2 for 10 days.

When the hybridmer reached to the maximum cell density, the amount of the IgM of the OAL- TFN - 01 within the culture supernatant was about 5 μg/nl.

(3) Titer of the antibody

The 96 well polystyrene micro-plate (manufactured by Nunc) coated with the purified product of the ED-B maintaining FN originated from placenta (after placenta was homogenized, it was extracted with urea) at 2 μg/well (at 4 °C for 24 hours) was

blocked with Dulbecco's phosphate buffer solution (pH 7.2, below abbreviated as D' PBS) at 4 °C for 24 hours. Then, each well of the said plate was added with 50 µl of the culture supernatant comprising the antibody of the present invention obtained in Example 2 and reacted at room temperature for three hours. After washing with the washing buffer solution (D' PBS + 0.05% Tween 20) for three times, the antibody bound to the fused protein between the ED-B of FN and protein A was measured by using peroxidase labeled sheep anti-mouse immunoglobulin antibody (manufactured by Zymet).

As a result, sufficient coloration was recognized when the said culture supernatant was diluted to 1×10^1 times.

(4) Standard curve by the ELISA method

The monoclonal antibody of the present invention was diluted with D' PBS to 25 µg/nl, and this was placed to each well of the 96 well micro-plate by 100 µl and solidified once at 4 °C. Then, washed with D' PBD (comprising 0.05% Tween 20). Then, each well was added with 300 µl each of D' PBS, 0.05% thimerosal, and 0.5% bovine serum albumin (BSA) and the blocking was carried out at 4 °C for one night. After blocking, it was washed with D' PBS (comprising 0.05% Tween 20) and each well was added with 100 µl of 0.01 M phosphate buffer solution [0.1% NP-40 (Nonident P-40, manufactured by Sigma), 0.05% thimerosal, and 10% FCS, pH 5.5]. Further, each well was added with 20 µl each of FN purified from human plasma (pFN) and cancerous FN (cFN) purified from the culture supernatant of the cell WI- 38 VA 13 which is the human normal fibroblast WI-38 deteriorated by the tumor virus, both of which were diluted to various concentration, and incubated at room temperature for 2.5 hours. Then it was washed with D' PBD comprising 0.05% Tween 20 for six times.

Further, each of the said well was added with: biotinylated anti- FN monoclonal antibody ["OAL- pF115", pFN established by Sigma as the antigen, see "Clinical Pathology", Vol. 35, additional volume, (1987), p119; and The 18th Congress of the International Association of Medical Laboratory Technologists, Abstracts, p225 (1988)] (x 1000 times diluted solution to 100 µl /well), D' PBS (100 µl/well), 0.1% CHAPS (3-[(3- cholamido propyl) dimethyl ammonio]- 1- propane sulfonate), 0.1% BSA, 0.05% thimerosal solution; and 100 µl of the A buffer solution, then incubated for 2.5 hours, and washed with D' PBD comprising 0.05% Tween 20 for six times.

Then, 100 µl/well of avidin- peroxidase composite (manufactured by Bio- Rad) was added after being dissolved into the A buffer solution and incubated for an hour. After washing the plate with the washing buffer solution, 100 µl of o-phenylene diamine solution (OPD solution) was added per well and reacted at room temperature for 10 minutes. Then, the reaction was halted by adding 100 µl of 2 N sulfuric acid and the absorbance at 492 nm was measured.

The said result is shown in Figure 1.

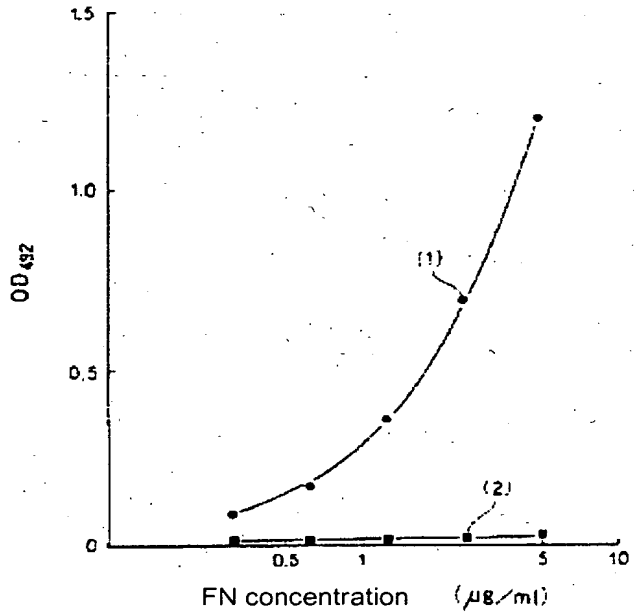
In this figure, the ordinate indicates the absorbance (OD) at 492 nm and the abscissa indicates the FN concentration, and (1) and (2) are the results for the cancerous FN and the plasma type FN, respectively.

The said figure apparently indicates that the antibody of the present invention does not react with the plasma type FN but reacts with the cancerous FN in the amount dependent manner

[Simple Interpretation of Figures]

Figure 1 is the graph presenting the reactivity of the antibody of the present invention towards various FNs.

Figure 1



Notes from the Translator

1. P. 594, bottom right column, line 5 and the rest of the specification (Page 11, middle)
   The units here are not very clear, thus nl is used.

2. P. 599, bottom right column, line 17 (p. 17, bottom)
   This was "sensitized cells" in JP H2-76598.

3. P. 600, upper right column and the rest, line 2 (P. 18 top)
   This superscript is illegible and may be 6 or 8.